Journal of Cardiovascular Pharmacology™
22:526-533 © 1993 Raven Press, Ltd., New York

EXHIBIT 9

Superior Activity of a Thromboxane Receptor Antagonist as Compared with Aspirin in Rat Models of Arterial and Venous Thrombosis

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Summary: We determined the effects of aspirin and a novel thromboxane A_2/prostaglandin endoperoxide (TP)-receptor antagonist, BMS-180291, on thrombosis and bleeding times in skin and mesenteric arteries. In anesthetized rats, occlusive thrombosis was induced in the carotid artery by topical application of ferrous chloride and in the vena cava by blood flow stasis combined with either infusion of thromboplastin or hypotonic saline. Aspirin (1, 10, and 50 mg/kg) did not reduce arterial or venous thrombus weight significantly. BMS 180,291 (150 μg/kg/min) decreased arterial thrombus weight and hypotonic saline-induced caval thrombus weight by 58 and 57%, respectively. BMS-180291 lacked antithrombotic activity at a lower dose (50 μg/kg/min) and failed to inhibit thromboplastin-induced caval thrombosis. BMS-180291 (150 μg/kg/min) significantly reduced arterial thrombus weight by 40% when plasma epinephrine concentration was increased to 5 ng/ml. BMS-180291 and aspirin produced increases of only ≤30% in bleeding times. These results demonstrate that BMS-180291 has antithrombotic activity in experimental aspirin-resistant arterial and venous thrombosis. Both aspirin and BMS-180291 have only modest effects on small artery hemostasis in rats.
Key Words: Thromboxane A_2 antagonist—Aspirin—Thrombin inhibitor—Arterial and venous thrombosis—Thromboplastin—Bleeding time—Rat.

Aspirin is the most commonly used antithrombotic drug in the United States and the only one that rapidly inhibits platelet function after oral administration. Aspirin is regarded as weakly antithrombotic, but it is the benchmark against which emerging drugs are tested (1). BMS-180291 is a selective thromboxane A_2/prostaglandin endoperoxide (TP) receptor antagonist (2) that produces prolonged inhibition of platelet function after oral administration to monkeys (3). We previously demonstrated partial inhibition of arterial thrombosis in rats with either aspirin or a TP-receptor antagonist (4). To differentiate better between aspirin and BMS-180291, we developed an aspirin-insensitive arterial thrombosis model adapted from that of Kurz and co-workers (5). This model uses topical application of iron chloride to induce formation of thrombi that consist predominantly of platelets and fibrin (5). The irreversible thrombin active-site inhibitor D-phenyl alanyl-L-prolyl-L-arginyl chloromethyl ketone (PPACK) (6,7) was tested in this model to provide a reference drug that inhibits both the platelet and coagulation components of thrombosis.

Platelets have a primary involvement in arterial thrombosis and also participate in venous thrombosis by accelerating thrombin formation (8). Millet and colleagues (9) combined mild vena cava injury in response to high-pressure saline infusion and blood flow stasis to induce thrombosis in rats that is highly sensitive to heparin. We previously observed that TP-receptor antagonists, but not aspirin, are partially effective in this model (10). Tissue factor (thromboplastin) activation of the extrinsic coagulation cascade has been proposed to be the predom-

Received December 14, 1993; revision accepted May 27, 1993.
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inant initiator of hemostasis in veins (11). We combined stasis of caval blood flow with thromboplastin injection for better testing of aspirin and BMS-180291 in clot formation initiated by this extrinsic pathway.

Because internal hemorrhage is a major side effect of antithrombotic therapy, doses of aspirin and BMS-180291 used in the thrombosis studies were evaluated in the small mesenteric artery bleeding model of Zawilska and associates (12) and also in a toe pad bleeding time model. Selection of BMS-180291 doses for all experiments was based on ex vivo inhibition of platelet reactivity to the TP-receptor agonist U-46,619. Aspirin doses were selected to bracket 10 mg/kg, a dose which in preliminary experiments blocked 99% of maximal thromboxane B_2 (TXB_2) production during clotting of rat blood.

METHODS

Arterial thrombosis

Male Sprague Dawley rats weighing 350–450 g were anesthetized intraperitoneally (i.p.) with Na-pentobarbital (50 mg/kg). Jugular veins were cannulated with PE-20 or PE-50 tubing for drug administration or anesthetic supplementation, and the trachea was intubated to ensure airway patency. The right carotid artery was exposed, and a piece of parafilm "M" was placed under the vessel. An electromagnetic flow probe (0.95- or 1.0-mm lumen) was placed on the artery and attached to a model MDL 1401 flowmeter (Skalar, Delft, Netherlands). After baseline flow measurements were made, a 2- × 5-mm strip of filter paper was saturated in a 50% solution of $FeCl_2$ and placed on top of the vessel downstream from the flow probe for 10 min. Some preliminary experiments were performed with a 50% solution of $FeCl_3$. The carotid artery was removed either on occlusion or 60 min after filter paper application if patency was maintained. The artery was opened lengthwise under a stereomicroscope, and the white thrombus was removed. Thrombus wet weight was determined immediately on a Sartorius R-160P balance (Brinkmann Instruments, Westbury, NY, U.S.A.). Carotid blood flow (CBF) was monitored continuously, and total CBF was calculated by planimetry and normalized as percentage of baseline (0 min) flow for 60 min.

Treatments were given intravenously (i.v.) starting 15 min before $FeCl_2$ application. The first study included vehicle, aspirin (1, 10, and 50 mg/kg) (Sigma Chemical, St. Louis, MO, U.S.A.) and BMS-180291 (50 and 150 μg/kg/min). The vehicles used in these and all other experiments were warm water for aspirin and a 10% solution of 95% ethanol plus 0.2% Na_2CO_{30} for BMS-180291 (25 μl/min). In the second study, a continuous infusion of epinephrine (Sigma) was given at a dose of 1.25 or 2.5 μg/kg/min in combination with vehicle or BMS-180291 (150 μg/kg/min). Each treatment consisted of at least 5 rats (exact numbers shown in Figs. 1 and 2). A separate group of rats not subjected to thrombosis received epinephrine infusions (2.5, 5 or 12.5 μg/kg/min; n = 4 per dose) while arterial blood pressure (BP) was monitored. Blood samples were obtained after 30 min of epinephrine infusion and frozen. Epinephrine levels were determined by Damon Clinical Laboratory (Trevose, PA, U.S.A.) using high-pressure liquid chromatography (HPLC) and electrochemical detection.

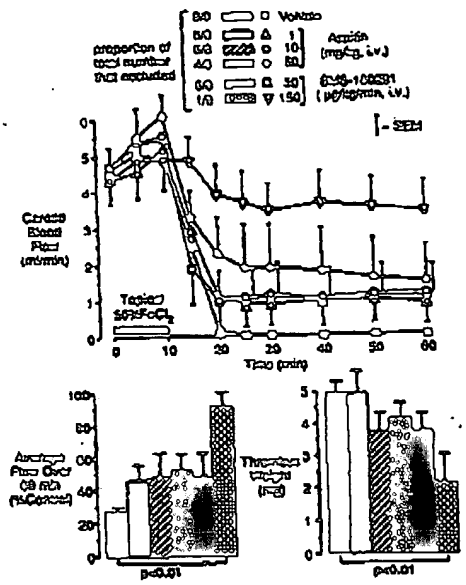

FIG. 1. Effects of aspirin and a thromboxane A_2/prostaglandin endoperoxide (TP)-receptor antagonist on arterial thrombosis were determined in anesthetized rats. Differences in thrombosis weight and blood flow averaged throughout the experiment were detected by analysis of variance only between vehicle and BMS-180291 (150 μg/kg/min). There were no significant differences among treatments in control (0 min) blood flow. Only BMS-180291 (150 μg/kg/min) reduced frequency of occlusion ($p < 0.05$, Fisher exact test).

A third study was performed using PPACK (Calbiochem, La Jolla, CA, U.S.A.) to provide a comparison with a irreversible thrombin inhibitor. Saline vehicle (n = 9) or PPACK (52 and 104 μg/kg/min; n = 7 and 9, respectively) was administered as described previously (Fig. 3). The PPACK doses are equivalent to 100 and 200 nmol/kg/min and were chosen from previous studies that demonstrated maximal inhibition of arterial thrombus formation in rats and monkeys (6,7).

Venous thrombosis

Male Sprague Dawley rats were anesthetized, the trachea was intubated, and the jugular vein was cannulated as described above. The vena cava was isolated by a midline abdominal incision. Caval thrombosis was induced by two different procedures. In the first procedure, a caval sac was produced by tying a ligature around a 26-gauge hypodermic needle just distal to the renal veins and applying a microaneurysm clamp just proximal to the bifurcation of the femoral veins. Another 26-gauge needle was inserted in the inferior portion of the venous sac, and hypotonic saline (0.225%) was infused at 10 ml/min for 15 s. This needle was removed after the saline flush, and the hole was sealed with a drop of cyanoacrylate cement. The proximal needle was then slipped free from the ligature, leaving a fixed nonocclusive stenosis, and the distal vas-

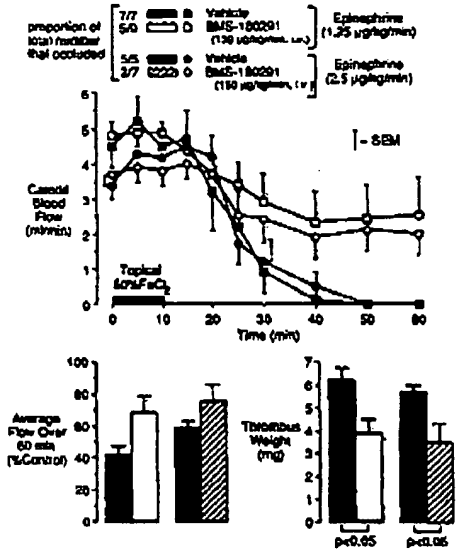

FIG. 2. Epinephrine was infused at two doses in an attempt to reverse the antithrombotic activity of BMS-180291 in anesthetized rats. Vehicle and BMS-180291 did not differ in either control (0 min) or averaged blood flow (*t* test). In rats receiving the 2.5-μg/kg/min infusion of epinephrine, frequency of occlusion was reduced by BMS-180291 ($p < 0.05$, Fisher exact test). BMS-180291 significantly reduced thrombus weights at both doses of epinephrine (*t* test).

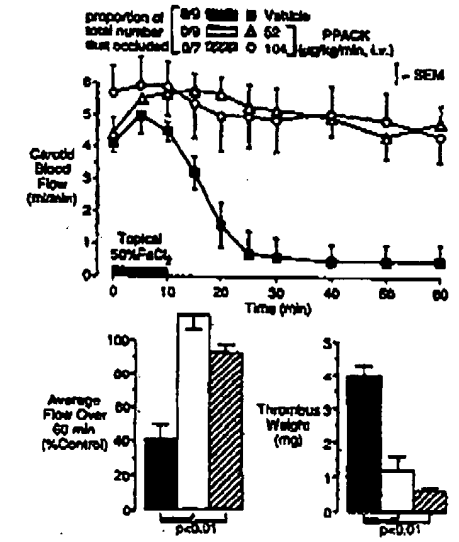

FIG. 3. Effect of the thrombin active-site inhibitor D-phenyl alanyl-L-prolyl-L-arginyl chloromethyl ketone (PPACK) on arterial thrombosis was determined in anesthetized rats. Significant reductions in thrombus weight and improvements in average blood flow were detected for both doses of PPACK by analysis of variance. PPACK also reduced frequency of occlusion ($p < 0.05$; Fisher exact test).

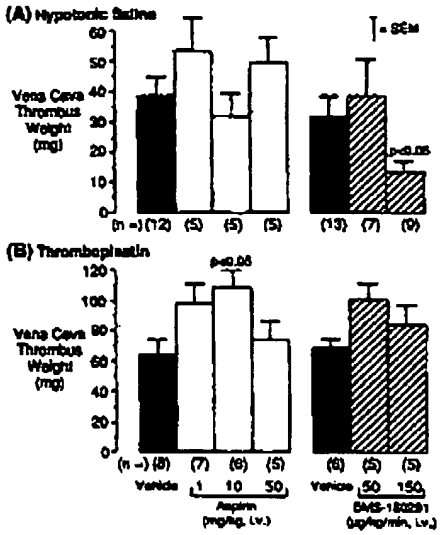

FIG. 4. The effects of aspirin and a TP-receptor antagonist on caval thrombus weights were determined in anesthetized rats. Thrombosis was induced by stasis of vena cava flood flow and infusion of either hypotonic saline (A) or thromboplastin (B). Comparisons with respective vehicle groups were made by analysis of variance.

cular clamp was removed. Blood flow was maintained through the stenosis for 20 min before proximal and distal vascular clamps were attached to reconstruct the caval sac. The sac was dissected free and slit open lengthwise. The exposed thrombus was removed and weighed immediately on the R-160P balance. Treatments were administered i.v. starting 15 min before the sac was constructed. There were two separate studies, consisting of vehicle and either aspirin (1, 10, and 50 mg/kg) or BMS-180291 (50 and 150 μg/kg/min). At least 5 rats were in each treatment group (exact numbers shown in Fig. 4).

In the second procedure, a caval stenosis was produced with a 26-gauge needle as described above; instead of hypotonic saline, thromboplastin (5 mg/kg) was infused for 2 min through a femoral vein catheter. This thromboplastin (no. T0263, Sigma) was derived from rabbit brain tissue with no added Ca^{2+}. In preliminary experiments, thromboplastin 5-mg/kg i.v. did not produce significant changes in circulating platelet or leukocyte counts, whereas higher doses (10–30 mg/kg) elicited thrombocytopenia and leukopenia. The thrombus was removed and weighed 20 min after thromboplastin injection as described above. Drug treatments were the same as those used in the first procedure and were administered 15 min before thromboplastin.

Mesenteric artery and toe bleeding times

Male Sprague-Dawley rats were anesthetized, the trachea was intubated, and the jugular vein was catheterized as described above. To measure mesenteric bleeding time the jejunum was isolated through a midline laparotomy and exteriorized. Small arteries branching perpendicular from the mesenteric artery over the surface of the jejunum were visualized by stereomicroscope. Individual arteries were punctured with a 30-gauge hypodermic needle

while the intestinal surface was superfused with Ringer's solution warmed to 37°C. To measure skin bleeding, an incision 5 mm long by 1 mm deep was made in the front paw toe pad with a Surgicutt instrument (International Technidyne, Edison, NJ, U.S.A.), and surgical gauze was used to absorb the blood without touching the wound. Bleeding times were determined by cessation of the free-flowing blood from the arterial or skin wound sites. Bleeding times were obtained from three to five vessels or two toe pad incisions before (control) and 15 min after administration of either vehicle, aspirin (1, 10, and 50 mg/kg) or BMS-180291 (50 and 150 μg/kg/min). Control and posttreatment bleeding times in each rat were averaged to single control and posttreatment values. There were at least 5 rats in each treatment group (exact numbers shown in Fig. 5).

Platelet reactivity and clotting times

Male Sprague Dawley rats were anesthetized, the trachea was intubated, and the jugular vein was cannulated as described above. To measure platelet reactivity, vehicle (n = 9) or BMS-180291 50 μg/kg/min (n = 3) and 150 μg/kg/min (n = 4) was infused i.v. for 35 min; arterial blood was then withdrawn into a 1/10 final volume of 3.8% Na-citrate. Blood samples were spun for 2 s in an Eppendorf 5412 microcentrifuge (Brinkman Instruments, Westbury, NY, U.S.A.) to prepare platelet-rich plasma (PRP) and again for 2 min to prepare PPP, which was used to adjust the platelet count to ~300 × 10^3 platelets/μl. Platelet shape change and aggregation responses to U-46,619 (0.01–100 μM, BioMol Laboratories, Philadelphia, PA, U.S.A.) were determined photometrically in a model 400VS platelet aggregometer (Chrono-Log, Havertown, PA, U.S.A.) using PPP to establish 100% aggregation. The ex vivo platelet shape change response was used to measure fractional TP-receptor inhibition. U-46,619 concentrations producing a 50% maximal shape change (EC_{50}) were determined, and concentration ratios (CR) were calculated (EC_{50} with BMS-180291/EC_{50} with vehicle). Percentage of TP-receptor inhibition was estimated from the formula [(CR-1)/CR] × 100 (13). The concentration-dependent inhibition of U-46,619–induced platelet shape change response by BMS-180291 was also characterized in vivo using platelet-rich plasma obtained from untreated rats.

We determined the in vitro effects of BMS-180291 on the activated partial thromboplastin time (APTT) and the prothrombin time (PT) using platelet-poor plasma (PPP) prepared as described above from untreated rats. The APTT and PT were measured using commercially available reagents (Baxter Healthcare, Miami, FL, U.S.A.) in the presence of varying BMS-180291 concentrations (0 to 10 μM). Clotting times were performed in triplicate at 37°C with a Fibrometer mechanical coagulation timer (Baxter Healthcare).

Statistical Analyses

Analysis of variance with Dunnett's test was used for multiple-group comparisons to vehicle. Other mean comparisons were made by contrasts. Baseline CBF was added as a covariate in analysis of average CBF. Two-way comparisons were made by *t* test, and the Fisher exact test was used for frequency of occlusion data. Bleeding times were expressed as percentage of change from control and were analyzed by analysis of covariance with control bleeding time as the covariate. Computations were performed using Systat software (Evanston, IL, U.S.A.). All data are mean ± SEM; $p < 0.05$ was considered significant.

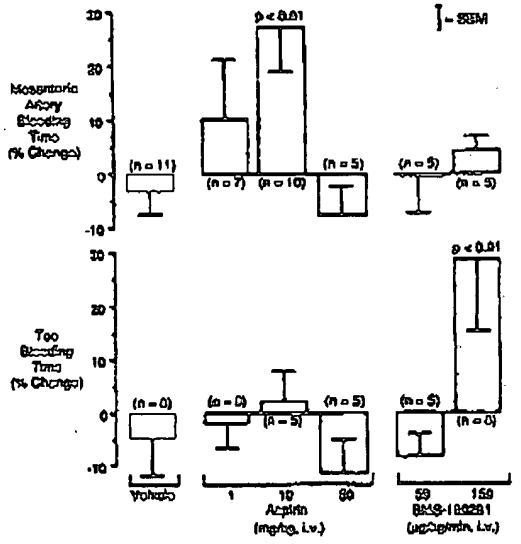

FIG. 5. Effects on aspirin and a TP-receptor antagonist on bleeding times in small mesenteric arteries and front leg toe pad were determined in anesthetized rats. Mesenteric bleeding times in response to vessel puncture were determined in three to five arteries before (control) and after treatment. Duplicate template bleeding times were determined in separate rats before and after treatment. Relative changes in bleeding time were compared with vehicle by analysis of variance with the control bleeding time used as a covariate. Control mesenteric bleeding times did not differ significantly among treatments and averaged 85 ± 2 s overall (n = 44). Neither did control toe bleeding times differ significantly among treatments, averaging 87 ± 2 s overall (n = 40).

RESULTS

Arterial thrombosis

Because preliminary experiments found that thrombus weights were smaller in response to $FeCl_3$ (2.5 ± 0.3, n = 15) as compared with $FeCl_2$ (5.1 ± 0.7, n = 7; $p < 0.01$, *t* test), $FeCl_2$ was used for further studies. Occlusive thrombosis was observed in all 9 vehicle-treated rats in the aspirin and BMS-180291 study, with CBF decreased to zero within 16 ± 1 min after removal of $FeCl_2$ (range 3–11 min). Aspirin at doses of 1, 10, and 50 mg/kg did not reduce average thrombus weight, improve patency, or stabilize CBF during thrombus formation (Fig. 1). There was a tendency for the 50-mg/kg dose of aspirin to improve average CBF, but this did not achieve statistical significance. In contrast to aspirin, BMS-180291 significantly reduced thrombus formation. Although BMS-180291 at a dose of 50 μg/kg/min was inactive, at 150 μg/kg/min it decreased average thrombus weight by 57%, in-

creased vessel patency to 88%, and maintained CBF at 91% of control after $FeCl_2$ exposure (Fig. 1).

In addition to drug effects on thrombus weight, there were also qualitative differences in thrombus appearance. Thrombi from vehicle-treated rats appeared under the stereomicroscope as predominantly white masses with interspersed dark red regions. We easily removed these thrombi intact by gently pulling the firm clot away from the site of $FeCl_2$ application. Thrombi from the BMS-180291-treated rats differed in that they were less firm and often disintegrated during removal. A similar difference in thrombus consistency was occasionally observed in aspirin-treated rats.

We attempted to reverse the antithrombotic activity of BMS-180291 by increasing plasma epinephrine to high physiologic levels. Preliminary experiments identified the appropriate epinephrine doses. Thirty-minute infusions of epinephrine at 2.5, 5, and 12.5 μg/kg/min produced proportionate increases in its plasma concentration to 5.0 ± 1.0, 10.4 ± 0.6 and 22.5 ± 4.1 ng/ml, respectively (n = 4 per dose). Mean arterial BP (MAP) was unaffected by the 2.5-μg/kg/min dose (124 ± 3 to 121 ± 9 mm Hg), but was increased by the 5- and 12.5-μg/kg/min doses (124 ± 5 to 150 ± 6 and 120 ± 10 to 140 ± 9 mm Hg, respectively; $p < 0.05$). Because the 2.5-μg/kg/min epinephrine dose produced high plasma levels with no marked hemodynamic effect, it and a lower dose of 1.25 μg/kg/min were selected for thrombosis studies. BMS-180291 significantly reduced arterial thrombus weight by 37 and 40% when coadministered with the 1.25- and 2.5-μg/kg/min infusions of epinephrine, respectively (Fig. 2). In the presence as compared with the absence of catecholamine infusion, BMS-180291 was less effective in sustaining CBF and vessel patency.

The thrombin inhibitor PPACK reduced thrombus weight by 70 and 86% at doses of 52 and 104 μg/kg/min, respectively (Fig. 3). PPACK also completely prevented both vessel occlusion and reductions in CBF during thrombosis.

Venous thrombosis

Infusion of hypotonic saline combined with stasis of blood flow produced vena cava clots in all vehicle-treated rats with an average overall weight of 35.6 ± 4.2 mg (range 10–83 mg, n = 25). Aspirin at doses of 1, 10, and 50 mg/kg did not significantly affect caval clot weight (Fig. 4A). BMS-180291 produced dose-dependent inhibition of venous thrombosis in the same manner observed with arterial thrombosis. Average venous thrombus weight was reduced 58% by the 150-μg/kg/min dose of BMS-180291, but not by the 50-μg/kg/min dose (Fig. 4A).

Thromboplastin injection combined with blood flow stasis produced caval clots in vehicle-treated rats that averaged 68.7 ± 7.2 mg overall (range 45–99 mg, n = 12). The average weight of these clots was greater than those produced by hypotonic saline ($p < 0.01$, t test) and it was not affected by BMS-180291 (Fig. 4B). Aspirin produced either a small increase (10 mg/kg) or had no effect (1 and 50 mg/kg) on thrombus weight (Fig. 4B). In both venous thrombosis models, clots obtained from vehicle- and drug-treated rats appeared as solid and uniform, dark red masses.

Mesenteric artery and toe pad bleeding times

Bleeding times were not significantly affected by vehicle treatment; this indicates that both preparations were highly sensitive to drug effects. The 10-mg/kg dose of aspirin produced a slight but significant 27 ± 9% increase in mesenteric bleeding time (Fig. 5). Higher and lower doses of aspirin (1 and 50 mg/kg) and both doses of BMS-180291 (50 and 150 μg/kg/min) had no effect on mesenteric bleeding time. BMS-180291 (150 μg/kg/min) significantly increased toe pad bleeding time by 30 ± 14%, whereas aspirin (1, 10, and 50 mg/kg) and the lower dose of BMS-180291 (50 μg/kg/min) had no effect in this model (Fig. 5).

Platelet reactivity and clotting activities

PRP plasma obtained from all 9 vehicle-treated rats showed a concentration-dependent shape change response to U-46,619 (Fig. 6). BMS-180291 infusion shifted the ex vivo concentration-effect curve for platelet shape change to the right in a parallel manner. Increases in the EC_{50} values of U-46,619 produced by BMS-180291 doses of 50 and 150 μg/kg/min indicated 99 and 99.9% antagonism of TP-receptors responsible for the platelet shape change response, respectively. Aggregation was ob-

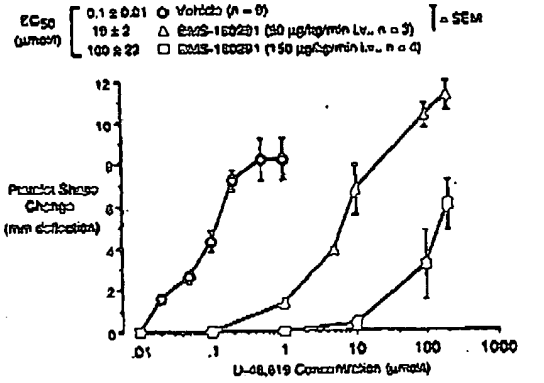

FIG. 6. Shape change response of rat platelets to U-46,619 was determined ex vivo after a 35-min infusion of either vehicle or BMS-180291. This was the amount of drug that had been administered at the times when thrombi were removed in the venous thrombosis model and when all vehicle-treated rats had occluded in the arterial thrombosis model. Shape change was measured as millimeters of deflection below the 0% aggregation baseline. Both BMS-180291 doses significant ($p < 0.05$) increased the EC_{50} value of U-46,619 (analysis of variance).

served in only 5 of 9 vehicle-treated rats, with a maximum response of 26 ± 5 mm at 10 μ*M* U-46,619 (80 mm = 100% aggregation). Both doses of BMS-180291 also completely blocked U-46,619-induced platelet aggregation.

The affinity of BMS-180291 for TP-receptors was also determined in vitro. The control EC_{50} for U-46,619-induced platelet shape change was 0.11 μ*M* and was increased to 1.8, 11.6, and 21.5 μ*M* by BMS-180291 concentrations of 30, 100, and 300 n*M*, respectively. Schild-plot analysis of this data showed a pA_2 of 8.7 with a slope of −1.10. This slope is not significantly different from unity and is consistent with competitive inhibition. The drug receptor dissociation constant (K_d) was 1.5 ± 0.29 n*M*.

Concentrations of BMS-180291 that were up to 5,000-fold greater than its K_d for inhibition of platelet TP-receptors did not inhibit either the intrinsic (APTT) or extrinsic (PT) clotting activity of rat plasma (Table 1). High concentrations of BMS-180291 (1 and 10 μ*M*) produced a slight yet significant, 13% decrease in PT.

DISCUSSION

Transition metals such as iron and copper are toxic in vivo because they induce lipid peroxidation (14). Kurz and co-workers (5) observed that topical application of $FeCl_3$ produces transmural injury in rat carotid arteries, resulting in occlusive thrombosis. We obtained similar results with $FeCl_2$, although thrombi were larger than those produced by $FeCl_3$. Aspirin did not inhibit $FeCl_2$-induced thrombosis at doses of 1, 10, and 50 mg/kg, although 10 mg/kg completely inhibited blood-derived cyclooxygenase. The preliminary experiments used to demonstrate 99% inhibition of TXB_2 production in whole blood in response to 10 mg/kg aspirin were performed under dosing conditions identical to those used in the present study. The 50-mg/kg dose of aspirin improved CBF slightly during thrombosis, suggesting that vasoconstrictor prostaglandins may limit flow in this model. Although 50 mg/kg is a high dose by clinical standards, tissue cyclooxygenase in rats is somewhat resistant to aspirin and optimum inhibition requires doses ≥30 mg/kg (15). Results of studies in which aspirin was used in rat models of arterial thrombosis have been mixed. Some investigators have reported that aspirin is ineffective at doses ranging from 3 to 300 mg/kg (16–20). Others have observed partial antithrombotic activity at doses of 3–30 mg/kg (4,21–25). In some of these rat studies, activity was lost or even reversed at doses ≥10 mg/kg (21–23).

TABLE 1. *Effect of BMS-180291 on in vitro clotting activities of rat plasma*

	BMS 180291 concentration (μ*M*)			
Clotting time	0	0.01	1.0	10.0
APTT (s) (n = 5)	23.3 ± 1.5	23.6 ± 1.8	23.0 ± 1.4	20.5 ± 0.0
PT (s) (n = 7)	14.4 ± 0.4	13.5 ± 0.3	12.5 ± 0.4*	12.5 ± 0.5*

APTT, activated partial thromboplastin time; PT, prothrombin time.

Clotting times were determined with a fibrometer. Data are mean ± SEM.

* Difference detected at $p < 0.05$ as compared with control (0 μ*M*) by analysis of variance.

There are several explanations for the variable antithrombotic activity of aspirin in vivo. The extent of vessel injury differs among experimental models and is likely to have impact on the efficacy of weak antiplatelet drugs. Aspirin probably requires near-complete inhibition of TXA_2 and prostaglandin endoperoxide production for maximal efficacy in the more severe thrombosis models. This degree of inhibition might be associated with decreases in prostacyclin and increases in salicylate levels that could interfere with its antiplatelet action (26). Other nonspecific effects of aspirin, such as increased platelet spreading onto vascular endothelium (23,27), may result from acetylation of enzymes other than cyclooxygenase, but some beneficial effects of aspirin, including inhibition of shear-induced platelet activation, appear to require high doses (28). These characteristics, along with individual variability in platelet and bleeding responses to aspirin (29), tend to obfuscate dose selection. In considering the efficacy of aspirin, one should recognize that not all lipids capable of activating TP-receptors are cyclooxygenase products; e.g., the recently discovered F_2-isoprostanes can activate TP-receptors (30), and these prostaglandinlike compounds can be produced by radical initiated reactions that do not require cyclooxygenase (31).

Studies with a variety of TP-receptor antagonists in rat models of arterial thrombosis have been positive (4,18–20,23) even when aspirin was inactive (18–20). We observed that BMS-180291 reduced occlusive thrombus weight by 58%, which was sufficient in most rats to maintain vessel patency and stabilize flow. This was less than the 86% reduction in thrombus weight obtained with the irreversible thrombin inhibitor PPACK. Other investigators have also shown that active-site thrombin inhibitors are more effective than TP-receptor antagonists in preventing platelet deposition onto endothelial-injured arteries (23) and collagen-coated surfaces (32). These two drug classes differ qualitatively in their effects on thrombogenesis. TP-receptor antagonists primarily inhibit platelet recruitment and fibrin- or platelet-dependent stabilization of the thrombus. Thrombin inhibitors share these activities and have the additional ability to impede platelet adhesion and thrombus initiation (23,32,33). The profile of activity for TP-receptor antagonists might be most appropriate for chronic treatment in which

vascular pathology requires that primary hemostasis be functioning yet blunted.

One concern germane to narrow-spectrum antithrombotic drugs is the potential for alternate pathways of platelet activation to surmount their inhibition. Epinephrine has been shown to reverse platelet inhibition by aspirin in vitro (34) and in vivo (35) and to reverse the antithrombotic activity of a combined TXA_2 synthetase/TP-receptor antagonist (36). High physiologic plasma concentrations of epinephrine of 1–2 ng/ml were required for these effects (35,36). In comparison, the plasma epinephrine concentration in humans reaches 0.4 ng/ml during heavy exercise, and peak levels of 1 ng/ml can occur during myocardial infarction (37). An increase in plasma epinephrine to 5 ng/ml diminished but did not prevent the antithrombotic activity of BMS-180291. The Folts model used by other investigators to demonstrate the epinephrine reversal phenomenon (35,36) involves continuous monitoring of cyclical thrombus formation. These thrombi are more rich in platelets than fibrin since they form in 3–4 min. In our experiments, platelet accumulation was not monitored continuously, and this could have been increased by epinephrine. Nevertheless, in BMS-180291–treated rats receiving epinephrine, the platelet mass did not organize into a firm occlusive clot.

The antithrombotic activity of the 150 μg/kg/kg infusion of BMS-180291 was associated with ≥99% fractional inhibition of TP receptors. The 50-μg/kg/min dose of BMS-180291 completely blocked ex vivo platelet aggregation responses to U-46,619, but this activity was insufficient to inhibit arterial thrombosis. We and other investigators (3,38) showed that TP-receptor agonists are less effective in aggregating rat platelets as compared with primate platelets but are equally effective in inducing the platelet shape change response. Platelet reactivity to other mediators is increased by the shape change, and this response to TP-receptor agonists may be as physiologically important as the direct aggregation response. Because platelet shape change occurs at low agonist concentrations that activate few TP receptors, near-complete receptor blockade is necessary to prevent the reaction. This may explain the high dose of BMS-180291 necessary to impede arterial thrombosis. Guinea pigs platelets more closely resemble human platelets in reactivity to TXA_2, and both TP-receptor antagonists and aspirin have greater antithrombotic potency in guinea pigs than rats (19). Thus, the rat model provides a relatively stringent test for comparison to humans.

Maximal TP-receptor blockade was also necessary to obtain inhibition of venous thrombosis in the model of Millet and colleagues (9). Using the same reasoning we applied to arterial thrombosis suggests that minimal TP receptor-dependent platelet activation is sufficient to enhance caval thrombosis. The partial efficacy of BMS-180291 and failure of aspirin to inhibit venous thrombosis agrees with results of our previous study (10). Neither drug limited thromboplastin-induced thrombosis; therefore, thrombogenesis induced by direct activation of extrinsic clotting does not necessarily require TP-receptor activation.

Skin template bleeding time in humans is prolonged by both aspirin (29) and TP-receptor antagonists (39), but this effect is not pronounced and may not be relevant because template bleeding times do not predict hemorrhage or reflect the risk of bleeding elsewhere in the body (40). We chose to measure mesenteric artery bleeding time in the hope that this would be more relevant to serious surgical bleeding. Mesenteric artery bleeding times were not affected by BMS-180291 and were only moderately increased by aspirin, which suggests that in rats small artery hemostasis is not dependent on TP receptors. Overall, these experiments demonstrate that BMS-180291 has antithrombotic activity in large blood vessels superior to that of aspirin and does not disrupt hemostasis in small arteries.

REFERENCES

1. Coller BS. Antiplatelet agents in the prevention and therapy of thrombosis. *Annu Rev Med* 1992;43:171–80.
2. Ogletree ML, Harris DN, Schumacher WA, Webb ML, Misra RN. Pharmacological profile of BMS 180,291: a potent, long-acting, orally active thromboxane A_2/prostaglandin endoperoxide receptor antagonist. *J Pharmacol Exp Ther* 1993;264:570–8.
3. Schumacher WA, Steinbacher TE, Youssef S, Ogletree ML. Antiplatelet activity of the long-acting thromboxane receptor antagonist BMS 180,291 in monkeys. *Prostaglandins* 1992;44:389–97.
4. Schumacher WA, Heran CH, Steinbacher TE, et al. Thrombin inhibition compared with other antithrombotic drugs in rats. *Thromb Res* 1992;68:157–66.
5. Kurz KD, Main BW, Sandusky GI. Rat model of arterial thrombosis induced by ferric chloride. *Thromb Res* 1990;60:269–80.
6. Kaiser B, Markwardt F. Experimental studies on the antithrombotic action of a highly effective synthetic thrombin inhibitor. *Thromb Haemost* 1986;55:194–6.
7. Hanson SR, Harker LA. Interruption of acute platelet-dependent thrombosis by the synthetic antithrombin D-phenylalanyl-L-prolyl-L-arginyy chloromethyl ketone. *Proc Natl Acad Sci USA* 1988;85:3184–8.
8. Suttie JW, Jackson CM. Prothrombin structure, activation, and biosynthesis. *Physiol Rev* 1977;57:1–70.
9. Millet J, Theveniaux J, Pascal M. A new experimental model of venous thrombosis in rats involving partial stasis and slight endothelium alterations. *Thromb Res* 1987;45:123–33.
10. Schumacher WA, Heran CL. Effect of thromboxane receptor antagonists on venous thrombosis in rats. *J Pharmacol Exp Ther* 1989;248:1103–15.
11. Rapaport SI, Rao LVM. Initiation and regulation of tissue factor-dependent blood coagulation. *Arterioscler Thromb* 1992;12:1111–21.
12. Zawilska KM, Born GVR, Begent NA. Effect of ADP-utilizing enzymes on the arterial bleeding time in rats and rabbits. *Br J Haematol* 1982;50:317–25.
13. Schumacher WA, Goldenberg HJ, Harris DN, Ogletree ML.

Effect of thromboxane receptor antagonists on renal artery thrombosis in the cynomolgus monkey. *J Pharmacol Exp Ther* 1987;243:460–6.
14. Halliwell B. Free radicals, reactive oxygen species and human disease: a critical evaluation with special reference to atherosclerosis. *Br J Exp Pathol* 1989;70:737–57.
15. Livid M, Benigni A, Zoja C, et al. Differential inhibition by aspirin of platelet thromboxane and renal prostaglandins in the rat. *J Pharmacol Exp Ther* 1989;248:334–40.
16. Reyers I, Mussoni L, Donati MB, de Gaetano G. Failure of aspirin at different doses to modify experimental thrombosis in rats. *Thromb Res* 1980;18:669–74.
17. Ashida S, Sakuma K, Abiko Y. Antithrombotic effects of ticlopidine, acetylsalicylic acid and dipyridamole in vascular shunt model in rats. *Thromb Res* 1980;17:663–71.
18. Massad L, Plotkine M, Capdeville C, Boulu RG. Electrically induced arterial thrombosis model in the conscious rat. *Thromb Res* 1987;48:1–10.
19. Takiguchi Y, Hirata K, Wada K, Nakashima M. Arterial thrombosis model with photochemical reaction in guinea-pig and its property. *Thromb Res* 1992;67:435–45.
20. Matsuno H, Uematsu T, Umemura K, Takiguchi Y, Wada K, Nakashima M. Effects of vapiprost, a novel thromboxane receptor antagonist, on thrombus formation and vascular patency after thrombolysis by tissue-type plasminogen activator. *Br J Pharmacol* 1992;106:533–8.
21. Philp RB, Paul ML, Killackey JJ, Killackey BA. The influence of dose, time of administration, body temperature and salicylate kinetics on the antithrombotic action of acetylsalicylic acid in male rats. *Haemostasis* 1983;13:42–52.
22. Parris J, Fournau P, Grancro M, Veins C. Antithrombotic effect of very low doses of acetylsalicyclic acid in rats. *Thromb Res* 1983;28:313–21.
23. Butler KD, Ambler J, Dolan S, Giddings J, Talbot MD, Wallis RB. A nonocclusive model of arterial thrombus formation in the rat and its modification by inhibitors of platelet function, or thrombin activity. *Blood Coag Fibrinolysis* 1992;3: 155–65.
24. Cooley BC, Li X, Dzwierzynski W, et al. The de-endothelialized rat carotid arterial graft: a versatile experimental model for the investigation of arterial thrombosis. *Thromb Res* 1992;67:1–14.
25. Meng K, Scuter F. Effect of acetylsalicylic acid on experimentally induced arterial thrombosis in rats. *Naunyn Schmiedebergs Arch Pharmacol* 1977;301:115–9.
26. Merino J, Livio M, Rajtar G, de Gaetano G. Salicylate reverses *in vitro* aspirin inhibition of rat platelet and vascular prostaglandin generation. *Biochem Pharmacol* 1980;29: 1093–6.
27. Rao GHR. Influence of anti-platelet drugs on platelet-vessel wall interactions. *Prostaglandins Leukotrienes Med* 1987; 30:133–45.
28. Ratnatunga CP, Edmondson SF, Rees GM, Kovas IB. High-dose aspirin inhibits shear-induced platelet reaction involving thrombin generation. *Circulation* 1992;85:1077–82.
29. Ryan WL, Hakenkamp K. Variable response to aspirin measured by platelet aggregation and bleeding time. *Lab Med* 1991;22:197–202.
30. Takahashi K, Nammour TM, Fukunga M, et al. Glomerular action of a free radical-generated novel prostaglandin, 8-epi prostaglandin-$F_{2\alpha}$ in the rat—evidence for interaction with thromboxane A_2 receptors. *J Clin Invest* 1992;90:136–41.
31. Morrow JD, Awad JA, Boss HJ, Blair IA, Roberts LJ. Non-cyclooxygenase derived prostanoids (F_2-isoprostanes) are formed *in situ* on phospholipids. *Proc Natl Acad Sci USA* 1992;89:10721–5.
32. Wagner WR, Hubbell JA. Evidence for a role in thrombus stabilization for thromboxane A_2 in human platelet deposition on collagen. *J Lab Clin Med* 1992;119:690–7.
33. Misselwitz F, Hofmann U, Leytin L, Repin VS. Influence of thromboxane receptor-antagonists on the interaction of platelets with solid-phase immobilized human collagen. *Biomed Biochim Acta* 1987;7:595–8.
34. Rao GHR, White JG. Epinephrine reverses the inhibitory influence of aspirin on platelet-vessel wall interactions. *Thromb Res* 1986;44:65–74.
35. Folts JD, Rowe GG. Epinephrine potentiation of in vivo stimuli reverses aspirin inhibition of platelet thrombus formation in stenosed canine coronary arteries. *Thromb Res* 1988;50:507–16.
36. Yao K-K, Rosolowsky M, Anderson HV, et al. Combined thromboxane A_2 synthetase inhibition and receptor blockade are effective in preventing spontaneous and epinephrine-induced canine coronary cyclic flow variations. *J Am Coll Cardiol* 1990;16:705–13.
37. Cryer PE. Physiology and pathophysiology of the human sympathoadrenal neuroendocrine system. *N Engl J Med* 1980;303:436–44.
38. Nakano T, Kamasaki K, Arita H. Role of protein kinase C in U46619-induced platelet shape change, aggregation and secretion. *Thromb Res* 1989;56:299–306.
39. de Boer A, Kroon JM, Kroon C, et al. Pronounced effects of the combination of a new thromboxane antagonist (GR32191) and heparin on bleeding time in man. *Thromb Haemost* 1992;68:24–9.
40. Rodgers RP, Levin J. A critical reappraisal of the bleeding time. *Semin Thromb Hemost* 1990;16:1–20.